RNA (1996), 2:213–225. Cambridge University Press. Printed in the USA.

The canonical GU dinucleotide at the 5′ splice site is recognized by p220 of the U5 snRNP within the spliceosome

JOSÉ L. REYES, PAVOL KOIS,[1] BOYANA B. KONFORTI,[2] and MARIA M. KONARSKA
The Rockefeller University, New York, New York 10021, USA

ABSTRACT

Specific recognition of the 5′ splice site (5′SS) by the spliceosome components was studied using a simple in vitro system in which a short 5′SS RNA oligonucleotide specifically induces the assembly of snRNP particles into spliceosome-like complexes and actively participates in a *trans*-splicing reaction. Short-range cross-linking demonstrates that a U5 snRNP protein component, p220 (the human analogue of the yeast Prp8) specifically interacts with the invariant GU dinucleotide at the 5′ end of the intron. The GU:p220 interaction can be detected in the functional splicing complex B. Although p220 has been known to contact several nucleotides around the 5′ splice junction, the p220:GU dinucleotide interaction described here is remarkably specific. Consistent with the high conservation of the GU, even minor modifications of this element affect recognition of the 5′SS RNA by p220. Substitution of uridine at the GU with base analogues containing a large methyl or iodo group, but not a smaller fluoro group at base position 5, interferes with association of 5′SS RNA with snRNP complexes and their functional participation in splicing.

Keywords: pre-mRNA splicing; p220/Prp8; 5′ splice site recognition; *trans*-splicing

INTRODUCTION

Pre-mRNA splicing takes place in a large, multicomponent complex, called the spliceosome. Spliceosome formation requires snRNP particles (U1, U2, U4, U5, and U6 snRNPs) and a number of non-snRNP protein factors and proceeds through a stepwise assembly process (reviewed in Moore et al., 1993). In addition, this process requires specific, conserved sequences in the pre-mRNA that include a consensus 5′ splice site (5′SS), a branch site with an adjacent polypyrimidine tract, and the 3′ splice site AG dinucleotide. Recognition of the 5′SS sequence is critical for spliceosome assembly. Initially, base pairing between the 5′ end of U1 snRNA and the 5′SS was shown to play an important role in determining the overall specificity of 5′SS selection (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988). However, this base pairing is disrupted subsequently and replaced by the interaction of the 5′SS with components of the U4/U5/U6 snRNP at later stages of spliceosome assembly. A specific base pairing interaction between the conserved ACAGAG sequence of U6 snRNA and intron bases at the 5′SS was demonstrated both by genetic and biochemical analyses (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993). Furthermore, exon sequences at the 5′SS are thought to interact with a conserved loop in U5 snRNA (Newman & Norman, 1991, 1992; Sontheimer & Steitz, 1993; Newman et al., 1995). However, the spliceosome components interacting with intron positions 1–4, including the most highly conserved element in the 5′SS, the GU dinucleotide, have so far not been identified.

In addition to snRNA:pre-mRNA interactions, a number of protein factors interact with splice site sequences (reviewed in Moore et al., 1993). In particular, several regulatory proteins, including members of the SR family, may be involved in the 5′SS:snRNP interactions (Ge & Manley, 1990; Krainer et al., 1990; Zahler et al., 1993). Furthermore, Stolow and Berget (1991) have identified two 5′SS-specific binding proteins. Finally, the U5 snRNP-specific yeast Prp8 protein and its

Reprint requests to: Maria Konarska, The Rockefeller University, 1230 York Avenue, New York, New York 10021, USA; e-mail: konarsk@rockvax.rockefeller.edu.
[1] Present address: Komensky University, Department of Organic Chemistry, Bratislava SK-84215, Slovakia.
[2] Present address: Columbia University, Department of Biochemistry and Molecular Biophysics, New York, New York 10032, USA.

mammalian analogue p220, can be crosslinked to pre-mRNA (Garcia-Blanco et al., 1990; Whittaker & Beggs, 1991). Site-specific crosslinking experiments indicate that this protein interacts with the 5′SS, branch site, polypyrimidine tract, and 3′SS region (Wyatt et al., 1992; MacMillan et al., 1994; Teigelkamp et al., 1995; Umen & Guthrie, 1995). Furthermore, p220 crosslinking to the intron lariat intermediate indicates that the interaction of p220 with the splice site regions persists beyond the first step of splicing (Wyatt et al., 1992; Teigelkamp et al., 1995; Umen & Guthrie, 1995).

Previously, we have described an in vitro system to analyze the spliceosome assembly pathway and interactions between the 5′SS and spliceosomal components (Hall & Konarska, 1992; Konforti et al., 1993). A short RNA oligonucleotide comprising the 5′SS consensus sequence (5′SS RNA oligo, AAG/GUAAGUAT, where / represents the exon/intron junction) can bind specifically to U4/U5/U6 snRNP, generating an intermediate complex that can be converted subsequently into a spliceosome-like U2/U4/U5/U6 snRNP complex upon addition of U2 snRNP. In both types of complexes, the 5′SS RNA can be crosslinked to U6 snRNA. The specificity of 5′SS recognition by U4/U5/U6 snRNP correlates with the 5′SS consensus sequence, suggesting that 5′ splice site recognition in vivo is also mediated by U4/U5/U6 snRNP components (Konforti & Konarska, 1994). Using this system, we show here that p220 recognizes the GU dinucleotide at the 5′ end of the intron, the most highly conserved element within the 5′SS sequence. This interaction is remarkably specific because substitution of the uridine residue within the GU with thymidine or 5-iodo-uridine diminishes the 5′SS RNA interaction with p220. This effect is correlated with a decreased splicing efficiency of the modified 5′SS RNA in a *trans*-splicing assay. Consistent with this finding, the p220:GU contact measured by short-range UV crosslinking can be detected within the functional splicing complex B.

RESULTS

p220 crosslinks to the 5′SS RNA oligo in U4/U5/U6 and U2/U4/U5/U6 snRNP complexes

UV-crosslinking methods were used to identify interactions between the 5′SS RNA oligo and components of the spliceosome. U2/U4/U5/U6 snRNP:5′SS RNA complexes were formed by incubating nuclear extract with 5′ end-labeled RNA oligo at 20 mM KCl on ice in the presence of the 5′SS DNA oligo to prevent association of the 5′SS RNA with U1 snRNP (Hall & Konarska, 1992; Konforti et al., 1993). The samples were then irradiated with 254 nm UV light, and the crosslinked material was resolved in a polyacrylamide SDS gel (SDS gel). Several protein:5′SS RNA crosslinks, resistant to RNase but eliminated by proteinase K digestion, were detected in these reactions (Fig. 1A, lanes 1, 2; Fig. 1B, lane 5 and data not shown). No signal was detected in samples incubated in the absence of nuclear extract or not subjected to UV irradiation (Fig. 1B, lanes 1, 2). Among the major RNase-resistant crosslink products was a set of proteins of ~50 kDa (Fig. 1A,B). These products were also observed in reactions containing

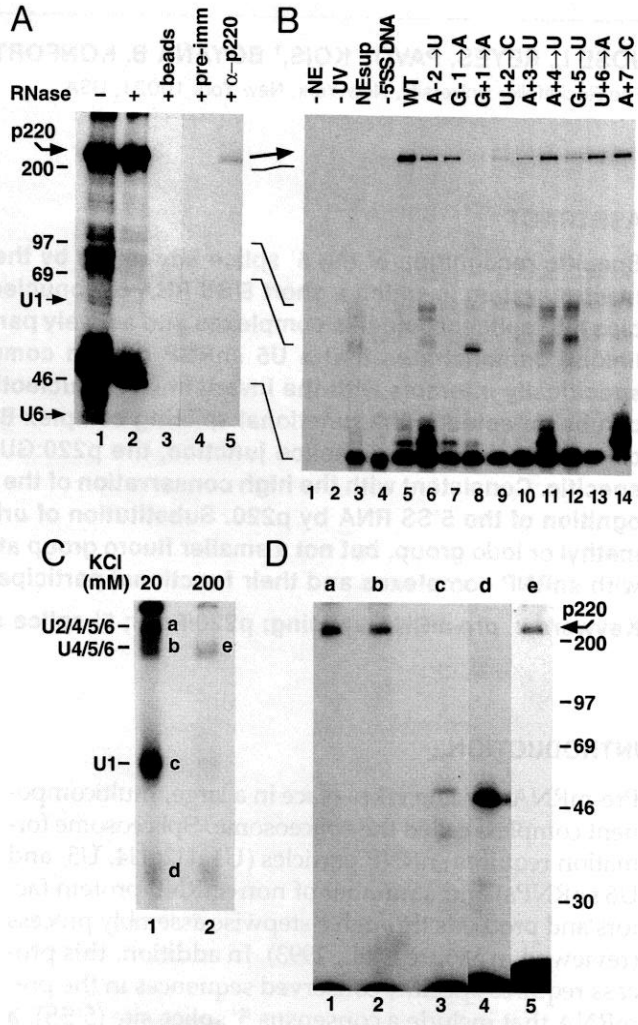

FIGURE 1. p220 crosslinks to the 5′SS in U4/U5/U6 and U2/U4/U5/U6 snRNP complexes. **A:** Binding reactions containing 5′ end-labeled wt 5′SS RNA were UV irradiated (254 nm light) and resolved in a 15% SDS gel directly (lane 1), or after additional RNase A treatment (lane 2). Alternatively, RNase A-treated reactions were subsequently immunoprecipitated using control beads (lane 3), pre-immune serum (lane 4), or anti-p220 serum (lane 5). Positions of the 5′SS RNA crosslinks to p220, U1, and U6 snRNA, as well as size markers, are indicated. **B:** Binding reactions containing 5′ end-labeled wt or mutant 5′SS RNA were UV irradiated and resolved in a 10% SDS gel. Reactions contained: Lane 1, no nuclear extract; lane 2, no UV irradiation; lane 3, snRNP-free nuclear extract supernatant; lane 4, no 5′SS DNA; lane 5, wt 5′SS; lanes 6–14, individually mutated 5′SS RNA oligos, as indicated. **C:** Binding reactions conducted in the presence of 20 or 200 mM KCl were UV-irradiated and resolved in a 4% nondenaturing gel. snRNP complexes marked a–e were excised from the nondenaturing gel and resolved in a second, 10% SDS gel shown in D, lanes 1–5, respectively. Positions of U2/U4/U5/U6 and U4/U5/U6 snRNP complexes are indicated.

snRNP-free supernatants of nuclear extracts and thus do not represent proteins associated with snRNP particles (Fig. 1B, lane 3). In contrast, a characteristic protein larger than 200 kDa was reproducibly detected in reactions containing nuclear extracts, but not their snRNP-free supernatants (Fig. 1B, lanes 5, 3). Formation of this abundant crosslink (~1% of the input 5′SS RNA oligo) was stimulated strongly in the presence of 5′SS DNA oligo, under conditions that favor association of U2/U4/U5/U6 snRNP:5′SS RNA complex (Fig. 1B, lanes 4, 5). In addition, two crosslinks described previously (Konforti et al., 1993), representing interactions of the 5′SS RNA oligo with U1 and U6 snRNAs, respectively, could be detected in these reactions. As expected, these snRNA:5′SS RNA crosslinks were eliminated by RNase A digestion (Fig. 1A, lane 1, 2).

p220 protein, a human analogue of the yeast Prp8 protein (Anderson et al., 1989; Pinto & Steitz, 1989; Garcia-Blanco et al., 1990), has been shown to reside in the U5 snRNP and to crosslink to both 5′ and 3′ SS regions in pre-mRNA (Wyatt et al., 1992; MacMillan et al., 1994; Teigelkamp et al., 1995; Umen & Guthrie, 1995). We used an antibody generated against the 50-kDa C-terminal fragment of human p220 (the generous gift of M. Moore and P.A. Sharp) to test the identity of the protein crosslinked to the 5′SS RNA. To this end, the binding reaction was subjected to UV irradiation, treated with RNase A to disrupt snRNP particles associated with the 5′SS RNA oligo, and incubated with anti-p220 antibody. The large protein crosslink was specifically immunoprecipitated by the anti-p220 antiserum (Fig. 1A, lane 5), but did not react with the resin or pre-immune serum (Fig. 1A, lanes 3, 4). This result shows that the 5′SS RNA oligo contacts p220 in nuclear extracts.

To investigate the specificity of 5′SS recognition by proteins in nuclear extracts, crosslinking profiles of 5′SS RNA oligos containing single-point mutations from position −2 to +7 were examined (Fig. 1B, lanes 6–14). The profile of 5′SS RNA oligo crosslinking to p220 in these reactions is remarkably similar to that of its binding to U4/U5/U6 and U2/U4/U5/U6 snRNP complexes (Konforti & Konarska, 1994). Furthermore, crosslinking properties of mutant oligos correlate with the degree to which positions −2 to +7 are conserved among natural 5′SS. For example, mutation of either of the first two positions of the intron, G+1 or U+2, most strongly reduced p220 crosslinking (5 and 11% of wt, respectively), whereas mutations at positions A−2, G−1, A+4, U+6, or A+7 had less of an effect (44, 44, 50, 52, and 71% of wt, respectively) and those at positions A+3 or G+5 showed an intermediate effect (20 and 22% of wt, respectively). Thus, p220 crosslinking to the 5′SS RNA oligo displays sequence specificity similar to that of U2/U4/U5/U6 snRNP complex formation.

Because p220 is a component of the U5 snRNP within the U4/U5/U6 snRNP particle, it was important to identify snRNP:5′SS RNA complexes containing the p220:5′SS RNA crosslink. We have shown previously that the U4/U5/U6 triple snRNP specifically recognizes the 5′SS RNA and that the resulting intermediate particle can interact subsequently with the U2 snRNP to form U2/U4/U5/U6 snRNP:5′SS RNA complex (Konforti & Konarska, 1994). The intermediate complex forms preferentially at 200 mM KCl in the snRNP binding reaction (Fig. 1C). Subsequent lowering of the KCl concentration allows joining of U2 snRNP, generating U2/U4/U5/U6 snRNP:5′SS RNA complex (data not shown). snRNP:5′SS RNA complexes formed at 20 and 200 mM KCl were UV irradiated and resolved in a nondenaturing gel (Fig. 1C). Gel slices containing individual complexes (marked a–e in Fig. 1C) were excised and resolved in a 10% SDS gel. The resulting profile of the 5′SS RNA:protein crosslinks is strikingly simple; complexes formed between the 5′SS RNA and U4/U5/U6 or U2/U4/U5/U6 snRNP complexes contain a single p220 crosslink (Fig. 1D, lanes 1, 2, 5). In addition, upon longer exposure of the autoradiogram, the U6 snRNA:5′SS RNA crosslink can also be detected in these complexes (data not shown). For comparison, analysis of crosslink products within U1 snRNP:5′SS RNA complex detected the expected 5′SS RNA:U1 snRNA interaction as well as a crosslink to a ~55 kDa protein (Fig. 1D, lane 3). Because purified U1 snRNP does not contain a known 55-kDa protein, the identified crosslink may represent an SR protein associated with the 5′SS:U1 snRNP particle (Kohtz et al., 1994). The abundant ~50-kDa protein crosslink was identified as a component of a complex that migrates in nondenaturing gels faster than U1 snRNP and does not appear to contain any snRNPs (Fig. 1D, lane 4). Finally, the p220 crosslink could not be detected in control reactions in which snRNP particles were disrupted by pretreatment with micrococcal nuclease (data not shown). Together, these results show that the p220 component of the U5 snRNP interacts with the 5′SS RNA within the spliceosome-like complexes. Furthermore, this interaction is established already at the stage of U4/U5/U6 snRNP binding and thus does not require the presence of U2 snRNP.

The p220 crosslink is located at the 5′ end of the intron

To precisely position the site of p220 interaction within the 5′SS RNA, a series of RNase digestions of the crosslinked species was performed. p220 crosslinked to the 5′ end-labeled 5′SS RNA (11 nt, p*AAG/GUAAGUAdT, where p* indicates ^{32}P group) retained the radioactive label upon digestion with the pyrimidine-specific RNase A, locating the crosslink within the sequence p*AAG/GU (Fig. 1A, lane 2; Fig. 2, lane 2). Because digestion with the G-specific RNase T1 resulted in loss of label (Fig. 2, lane 3), the crosslink must lie down-

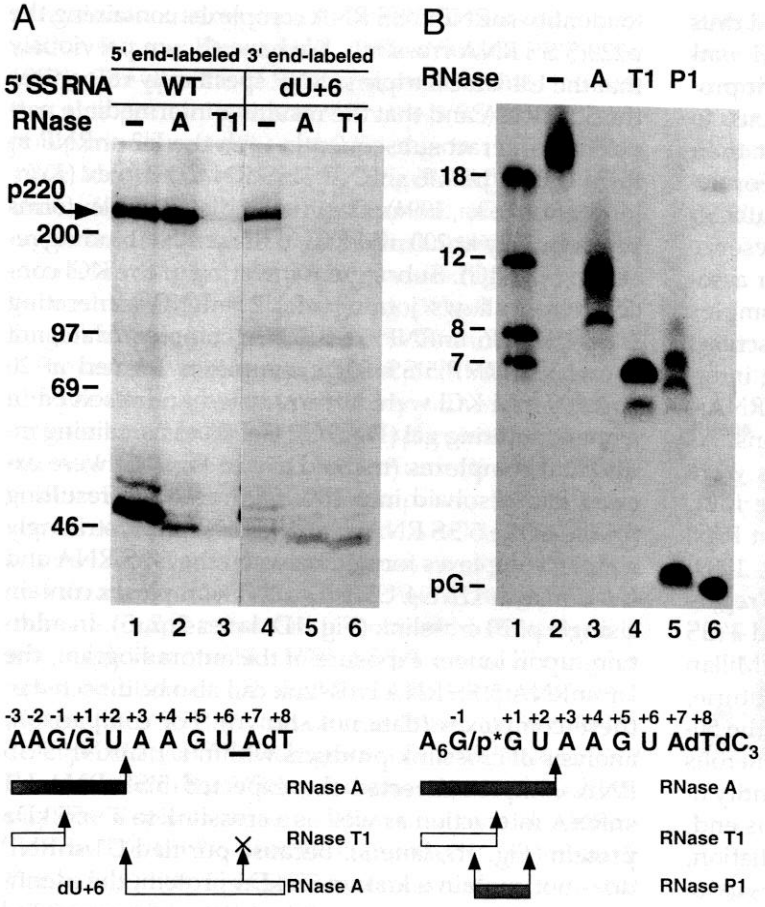

FIGURE 2. p220 crosslink maps to the conserved GU in the 5′SS. **A:** Binding reactions containing 5′ end-labeled wt 5′SS RNA oligo (11 nt, lanes 1–3) or 3′ end-labeled U+6 → dU 5′SS oligo (14 nt, lanes 4–6) were UV irradiated (254 nm) and digested with RNase A (lanes 2, 5) or RNase T1 (lanes 3, 6). Products were resolved in a 15% SDS gel. The position of p220 is indicated by an arrow, numbers indicate size (in kDa) of the molecular weight markers. **B:** Binding reactions conducted with 5′SS RNA ^{32}P-labeled at the exon/intron boundary (see lower panel) were UV-irradiated (254 nm) and resolved in a 7.5% SDS gel. p220:5′SS RNA crosslink product was excised from the gel, digested with proteinase K and resolved in a 20% polyacrylamide/8 M urea gel (lane 2). The same material was further digested with RNase A (lane 3), RNase T1 (lane 4), or nuclease P1 (lane 5). Products of partial digestion of the 5′ end-labeled 5′SS RNA oligo with RNase T1 (lane 1) and complete digestion with nuclease P1 (lane 6) served as size markers (in nt). pG is guanosine 5′-phosphate. Lower panels show diagram of the results. Dark bar indicates a 5′SS RNA fragment crosslinked to p220, open bars indicate uncrosslinked RNA fragments. The phosphodiester bond resistant to RNase cleavage due to the presence of deoxyribose at U+6 position is underlined. Asterisk indicates ^{32}P phosphate.

stream of the first G, i.e., within the sequence /GUAA GUAdT, and not in the 5′ exon *pAAG. When the 5′SS RNA oligo was labeled at the 3′ end by extension with three ^{32}P-dC residues, digestion of the crosslink with either RNase A or T1 resulted in a loss of signal (data not shown). Significantly, blocking the RNase A cleavage site at position U+6 by introduction of a deoxyU residue (U+6 → dU, see Fig. 3, bottom panel, for numbering of positions) also resulted in loss of signal (Fig. 2, lane 5), indicating that no additional crosslinks are located downstream of the GU dinucleotide. Together, these data constrain the site of p220 crosslink to the GU at the 5′ end of the intron.

To precisely identify the site of interaction, a 5′SS RNA oligo containing an internal ^{32}P label positioned at the exon/intron junction (A_6G/p*GUAAGUAdTdC$_3$, 18 nt) was used to generate the p220 crosslink (Fig. 2B). The 5′SS RNA used in these experiments contains a 7-nt rather than a 3-nt exon segment and three additional dC residues at the 3′ end. These changes in the oligo, however, do not affect its interaction with snRNPs (data not shown). The p220:5′SS RNA crosslink was isolated from a 7.5% SDS gel and further digested with proteinase K. The resulting oligopeptide: RNA crosslinks migrated slightly slower than the unreacted 5′SS RNA oligo in a 20% polyacrylamide/8 M urea gel (Fig. 2B, lane 2). Subsequent digestion of this material with RNase A yielded faster-migrating products (Fig. 2B, lane 3), whereas digestion with RNase T1 released a discrete, uncrosslinked RNA fragment with the mobility of the 7-nt exon segment (Fig. 2B, lane 4). Together, these results confirm that the site of crosslink is located within the GU dinucleotide. Finally, the same proteinase K-treated material was digested with nuclease P1, which generates 5′ nucleoside monophosphates (pN). Nuclease P1 treatment produced a heterogenous pattern characteristic of the crosslink products, in addition to some free pG, whereas the analogous digestion of the uncrosslinked 5′SS RNA yielded only the expected pG residue (Fig. 2B, lanes 5, 6). Thus, a significant fraction of the p220 crosslink induced by 254 nm UV maps directly to the G+1 at the 5′ end of the intron. The free pG released from the crosslink upon digestion with nuclease P1 could originate from molecules in which the crosslink between the 5′SS RNA and p220 occurred through U+2 rather than G+1 position. Alternatively, incubation conditions used for nuclease P1 digestion could destabilize the crosslink bond. This latter interpretation could also in part explain the low level of uncrosslinked RNA fragment detectable upon RNase A digestion of the same sample (Fig. 2B, lane 3).

Specific crosslinking of p220 to 4-thiouridine at the second position of the intron

An independent and direct confirmation of the site-specific interaction between the 5′SS RNA and p220 was provided by oligo mutants in which the Us at positions +2 and +6 were substituted individually with 4-thiouridine (4-thioU). The 4-thioU-substituted, 5′ end-labeled RNA oligos (11 nt) bind efficiently to U2/U4/U5/U6 snRNP complexes (Fig. 3C). In particular, U+2→4-thioU oligo formed U2/U4/U5/U6 snRNP:5′SS RNA complexes with an efficiency greater than that of the wt 5′SS RNA (121% of wt). snRNP:5′SS RNA complexes were irradiated with 365 nm UV light and crosslink products were analyzed in a 15% SDS gel (Fig. 3A). Under these conditions, crosslinks are formed specifically with the 4-thioU base (Bergstrom & Leonard, 1972; Lemaigre Dubreuil et al., 1991). As expected, no crosslinked products were detected when snRNP complexes containing unmodified, wt 5′SS RNA were irradiated with 365 nm light or when the crosslinking step was omitted (Fig. 3A, lanes 3, 1). When snRNP complexes formed with U+2→4-thioU 5′SS RNA oligo were irradiated with 365 nm UV light, the crosslinking profile was similar to that observed in reactions containing the wt 5′SS RNA irradiated with 254 nm light and included the characteristic p220 crosslink (Fig. 3A, lanes 2, 4). However, when the 4-thioU was placed at position U+6 in the 5′SS RNA, the p220 crosslink was significantly reduced, whereas the appearance of other crosslinked protein products was essentially unchanged (Fig. 3A, lane 5). These other crosslink products appear to be unrelated to splicing because they are also present in reactions containing snRNP-free extract supernatants (Fig. 3A, lane 6–8). Furthermore, the p220 crosslink formed using U+2 → 4-thioU 5′SS RNA is found within the U4/U5/U6 and U2/U4/U5/U6 snRNP complexes, whereas it is undetectable in complexes associated with U+6→4-thioU 5′SS RNA oligo (Fig. 3A, lanes 10–13). Independently, RNase A and RNase T1 mapping experiments confirmed the site of p220 crosslink at the predicted U+2→4-thioU position (data not shown).

In parallel, aliquots of the same reactions were deproteinized and analyzed in a 10% polyacrylamide-urea gel to detect snRNA:5′SS RNA crosslinks (Fig. 3B). As expected, crosslinking of the wt 5′SS RNA oligo to U1 and U6 snRNAs was detected. These crosslinks, formed upon irradiation with 254 nm, but not 365 nm UV light (Fig. 3B, lanes 1–3), are located typically near the ends of RNA duplexes (Sawa & Abelson, 1992; Konforti & Konarska, 1994). Interestingly, 365 nm light irradiation of snRNP complexes containing U+2 → 4-thioU 5′SS RNA oligo did not yield detectable crosslinks to either U1 or U6 snRNAs (Fig. 3B, lane 4). However, the analogous reactions with U+6 → 4-thioU 5′SS RNA oligo generated a very abundant (200% of wt at 254 nm) crosslink with U1 snRNA, which correlates with the efficient binding of this oligo to U1 snRNP (Fig. 3C, lane 5, 6), in addition to a less prominent, but clearly detectable (4% of wt at 254 nm) crosslink to U6 snRNA

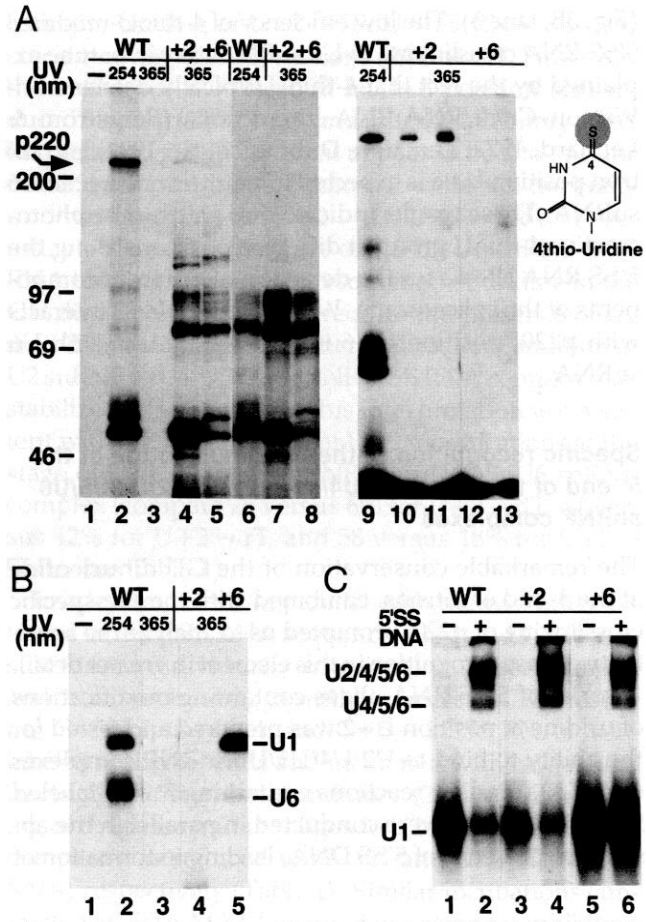

FIGURE 3. Specific crosslinking of p220 to 4-thiouridine at the second position of the intron. **A:** snRNP complexes formed in binding reactions containing wt 5′SS RNA oligo were not irradiated (lane 1), or irradiated with 254 nm UV light (lane 2) or 365 nm UV light (lane 3). Similarly, 5′SS RNA oligos containing 4-thioU at positions U+2 (lane 4) or U+6 (lane 5) were used in binding reactions and UV irradiated with 365 nm light. Alternatively, snRNP-free nuclear extract supernatant was incubated with wt 5′SS RNA and irradiated with 254 nm UV light (lane 6), or incubated with 5′SS RNA oligos containing 4-thioU at positions U+2 (lane 7) or U+6 (lane 8) and UV irradiated with 365 nm light. Crosslinked RNA:protein products were resolved in a 15% SDS gel. Binding reactions containing wt 5′SS RNA oligo, U+2→4-thioU or U+6→4-thioU were UV irradiated and resolved in a 4% nondenaturing gel. Complexes were excised from the gel and resolved in a second 10% SDS gel. Lane 9, complete binding reaction with wt 5′SS RNA oligo; lanes 10 and 12, U2/U4/U5/U6 snRNP complexes formed in the presence of U+2 → 4-thioU and U+6→4-thioU 5′SS RNAs, respectively; lanes 11 and 13, U4/U5/U6 snRNP complexes formed in the presence of U+2 → 4-thioU and U+6 → 4-thioU 5′SS RNAs, respectively. **B:** Reactions 1–5 from A were phenol extracted and RNA:RNA crosslink products resolved in a 10% polyacrylamide/8 M urea gel. **C:** Binding reactions performed in the absence (lanes 1, 3, 5) or presence (lanes 2, 4, 6) of 5′SS DNA oligo using wt 5′SS RNA oligo (lanes 1, 2), U+2 → 4-thioU (lanes 3, 4), or U+6→4-thioU (lanes 5, 6) were resolved in a 4% nondenaturing gel. Positions of molecular weight markers, 5′SS RNA crosslinks to U1 and U6 snRNAs, and 5′SS RNA:snRNP complexes are indicated. Chemical structure of the 4-thiouracil moiety of the nucleoside is shown.

(Fig. 3B, lane 5). The low efficiency of 4-thioU-modified 5′SS RNA crosslinking to U6 snRNA may in part be explained by the fact that 4-thioU typically detects non-Watson–Crick RNA:RNA interactions (Bergstrom & Leonard, 1972; Lemaigre Dubreuil et al., 1991) and intron position U+6 is expected to pair with nt A42 in U6 snRNA. These results indicate that placing the photoreactive 4-thioU group at different positions along the 5′SS RNA allows for the detection of different components of the spliceosome. While position U+2 interacts with p220, position U+6 is in close contact with U6 snRNA.

Specific recognition of the GU dinucleotide at the 5′ end of the intron by U4/U5/U6 and U2/U4/U5/U6 snRNP complexes

The remarkable conservation of the GU dinucleotide at the 5′ end of introns, combined with the site-specific crosslinking of p220, prompted us to analyze the specificity of base recognition in this element in greater detail. A series of 5′SS RNA oligos containing modifications of uridine at position U+2 was prepared and tested for the ability to bind to U2/U4/U5/U6 snRNP complexes (Fig. 4A). Binding reactions containing 5′ end-labeled 5′SS RNA oligos were conducted in parallel in the absence or presence of 5′SS DNA, leading to formation of U1 snRNP:5′SS RNA or U4/U5/U6 and U2/U4/U5/U6 snRNP:5′SS RNA complexes, respectively (Fig. 4B). Substitution of uridine with deoxythymidine (dT), U+2 → dT 5′SS RNA, resulted in a reduction (26% of wt) of U2/U4/U5/U6 snRNP:5′SS RNA complex formation (Fig. 4B, lane 4; Table 1). This inhibitory effect is not due to the presence of deoxyribose at this position because 5′SS RNA containing the analogous U+2 → dU substitution showed an enhanced level (129% of wt) of binding to U2/U4/U5/U6 snRNP complex (Fig. 4B, lane 6; Table 1). Similarly, 2′-O-methyl modification of ribose, U+2 → 2′-O-MeU, did not significantly affect binding properties of the 5′SS RNA (91% of wt, data not shown). Instead, the presence of a methyl group at position 5 of uridine, which represents the only difference between the T and U bases (Fig. 4A), must be responsible for the observed effect. Consistent with this prediction, the riboT modification, U+2 → rT, reduced the ability of the RNA oligo (44% of wt) to bind to U2/U4/U5/U6 snRNP complex (Fig. 4B, lane 8; Table 1). However, the same modification had no effect on binding (102% of wt) when placed 4 nt downstream, at position U+6 (Fig. 4B, lane 14; Table 1). One possible explanation for the above results is that steric hindrance caused by the bulky methyl group of thymidine (2.0 Å radius) interferes with the proper interaction of the base at oligo position +2 with p220. To test this

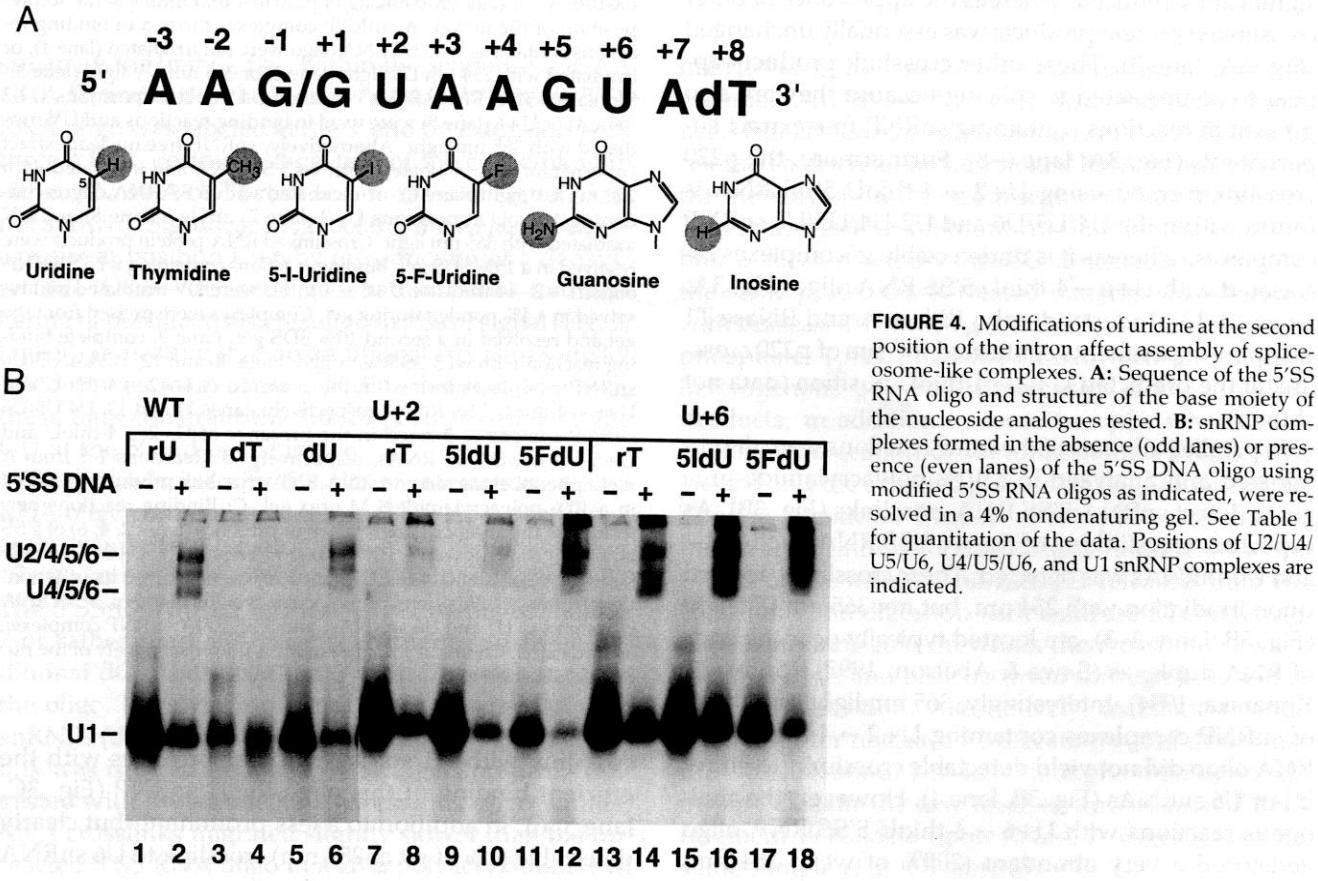

FIGURE 4. Modifications of uridine at the second position of the intron affect assembly of spliceosome-like complexes. **A:** Sequence of the 5′SS RNA oligo and structure of the base moiety of the nucleoside analogues tested. **B:** snRNP complexes formed in the absence (odd lanes) or presence (even lanes) of the 5′SS DNA oligo using modified 5′SS RNA oligos as indicated, were resolved in a 4% nondenaturing gel. See Table 1 for quantitation of the data. Positions of U2/U4/U5/U6, U4/U5/U6, and U1 snRNP complexes are indicated.

TABLE 1. Binding of selected 5′SS RNA mutants to U2/U4/U5/U6 and U4/U5/U6 snRNP complexes.

5′SS RNA	U2/U4/U5/U6 snRNP[a]	U4/U5/U6 snRNP[a]
wt	100 ± 15	100 ± 12
U+2 → A	5 ± 1	9 ± 4
U+2 → dT	26 ± 9	6 ± 1
U+2 → dU	129 ± 16	89 ± 22
U+2 → rT	44 ± 9	12 ± 4
U+2 → 5IdU	58 ± 10	18 ± 3
U+2 → 5FdU	102 ± 24	49 ± 16
U+6 → dU	88 ± 13	60 ± 3
U+6 → rT	102 ± 17	105 ± 23
U+6 → 5IdU	113 ± 9	136 ± 30
U+6 → 5FdU	108 ± 4	42 ± 10
G−1 → rI	69 ± 9	32 ± 5
G+1 → rI	56 ± 8	18 ± 4
G+5 → rI	20 ± 3	17 ± 2

5′SS RNA:snRNP complexes were resolved in a 4% nondenaturing gel. The efficiency of complex formation quantified by phosphorimager analysis was calculated as percent of total input 5′SS RNA bound to a snRNP complex. Numbers represent average and standard deviation in percent relative to wt, obtained from 3 to 10 independent experiments.

[a] Values for U2/U4/U5/U6 and U4/U5/U6 snRNP complexes were calculated from binding reactions conducted in the presence of 20 and 200 mM KCl, respectively.

possibility, we prepared a set of 5′SS RNA oligos in which position 5 of uridine was substituted with groups of increasing size. 5-Fluoro modification (5FdU), which substitutes hydrogen (1.2 Å) with fluorine (1.35 Å), does not affect 5′SS RNA interaction with U2/U4/U5/U6 snRNP complexes at either position U+2 or U+6 (102 and 108% of wt, respectively, Fig. 4B, lanes 12, 18; Table 1). In contrast, 5-iodo modification (5IdU) introduces iodine (2.15 Å), a substituent of a size similar to that of a methyl group (2.0 Å) present in thymidine. Although 5IdU modification does not affect 5′SS RNA:snRNP interaction when placed at position U+6 (113% of wt, Fig. 4B, lane 16; Table 1), it interferes with binding when present at position U+2 (58% of wt, Fig. 4B, lane 10; Table 1). Thus, the size of the substituting group at position 5 of uridine at the GU dinucleotide affects the interaction of the 5′SS RNA with multi-snRNP complexes.

In addition to standard binding reactions performed at 20 mM KCl, parallel incubations were conducted at 200 mM KCl to accumulate preferentially U4/U5/U6 snRNP:5′SS RNA complexes. Under these conditions, uridine modifications within the oligo that affect formation of U2/U4/U5/U6 snRNP:5′SS RNA complex have an even stronger effect on U4/U5/U6 snRNP:5′SS RNA association. In particular, U+2 substitution with rT reduced 5′SS RNA binding to 12% of wt. Similarly, U4/U5/U6 snRNP bound the U+2 → 5IdU 5′SS RNA with only 18% efficiency, whereas binding of U+6 → 5IdU 5′SS RNA was stimulated (136% of wt). Under the same conditions, binding of U4/U5/U6 snRNP to U+2 → A 5′SS RNA was reduced to 9% of wt (Table 1). Interestingly, binding of U+2 → 5FdU 5′SS RNA was less efficient under these conditions (49% of wt). However, the analogous modification at U+6 position also reduced binding (42% of wt), and thus the inhibitory effect of the fluoro group does not appear to be site-specific under these conditions. Because the effect of the bulky uridine substitutions (U+2 → dT, U+2 → 5IdU) is detectable already at the stage of U4/U5/U6 snRNP:5′SS RNA binding, the specific recognition of uridine in the GU dinucleotide does not require the presence of U2 snRNP in the complex. Rather, subsequent addition of U2 snRNP to U4/U5/U6 snRNP:5′SS RNA complex may stabilize this interaction. This interpretation is consistent with the lesser effects of U+2 modifications at the stage of U2/U4/U5/U6 snRNP than U4/U5/U6 snRNP complex (compare 26 versus 6% for U+2 → dT, 44 versus 12% for U+2 → rT, and 58 versus 18% for U+2 → 5IdU; see Table 1).

In addition, G+1, in parallel with the two other Gs found in the 5′SS RNA sequence, G−1 and G+5, was substituted individually with inosine (I, see Fig. 4A) and tested for binding to snRNP complexes. The resulting G−1 → I, G+1 → I, and G+5 → I 5′SS RNAs were labeled at their 5′ ends and incubated with nuclear extracts. Under standard conditions (20 mM KCl), binding of the three mutant oligos to U2/U4/U5/U6 snRNP complex was reduced to 69, 57, and 20% of wt 5′SS RNA, respectively (Table 1). Similar incubations conducted at 200 mM KCl resulted in a stronger inhibition of binding; formation of U4/U5/U6 snRNP:5′SS RNA complexes was reduced to 32, 18, and 17% of wt 5′SS RNA, respectively (Table 1). Thus, although inosine substitution affects binding of the 5′SS RNA to snRNP complexes, its effect is not restricted to a single position in the sequence.

Interaction between the 5′SS RNA and p220 within splicing complex B

To test whether p220 recognition of the invariant GU dinucleotide is maintained during the actual splicing reaction, we have used a functional *trans*-splicing assay (Konforti & Konarska, 1995) in which the 5′SS RNA oligo is spliced in *trans* to a longer (128 nt) Ad13 RNA containing the 3′SS region. In the presence of 5′SS DNA oligo, incubation of the internally labeled (A_6G/*pGUAAGUAdTdC$_3$) 5′SS RNA oligo and Ad13 RNA at 30 °C with nuclear extract produced two distinct RNA species identified as the branched intron/3′ exon intermediate and the branched intron product (Fig. 5A, lane 4). Aliquots of *trans*-splicing reactions resolved in nondenaturing gels showed the presence of large complexes associated with labeled 5′SS RNA oligo. U2/U4/U5/U6 snRNP:5′SS RNA complex is formed in the absence of Ad13 RNA upon incubation on ice (Fig. 5B, lane 2), but it is unstable at 30 °C (Fig. 5B, lanes 5, 6). In addition, a faster-migrating complex is generated

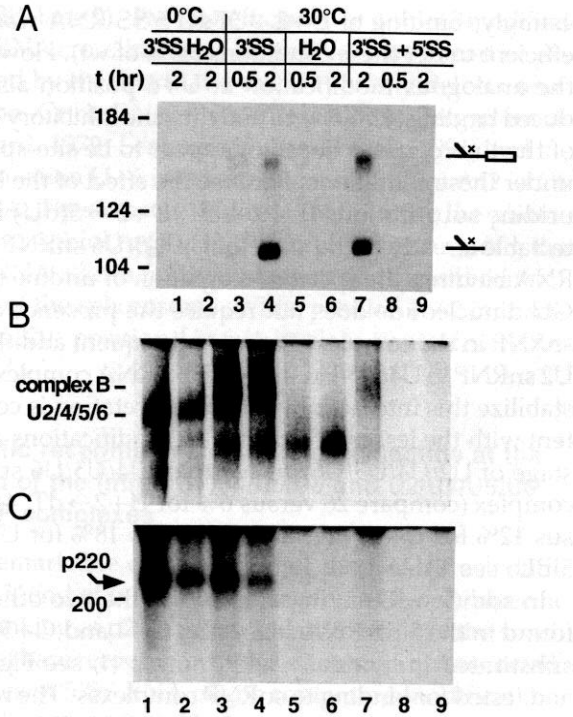

FIGURE 5. p220 interacts with the 5′SS RNA oligo within splicing complex B. Splicing reactions containing 5′SS RNA oligo labeled internally at the splice junction and 5′SS DNA oligo were incubated on ice (lanes 1, 2) or at 30 °C (lanes 3–9) in the presence (lanes 1, 3, 4, 7–9) or absence (lanes 2, 5, 6) of Ad13 RNA for the indicated period of time. For the chase experiment (lanes 7–9), Ad13 RNA was preincubated for 0.5 h at 30 °C to allow for complex A formation. Labeled 5′SS RNA was then added and, after 0.5-h incubation, a 1000-fold excess (100 ng) of unlabeled 5′SS RNA oligo was added and incubation continued for 1.5 h (lane 7). In lanes 8 and 9, the unlabeled and labeled 5′SS RNA oligos were added together and incubation continued for 0.5 and 2 h, respectively. **A:** Branched RNA intermediates and products of *trans*-splicing (indicated on the right) were resolved in a 10% polyacrylamide/8 M urea gel. Positions of DNA size markers (in nt) are indicated on the left. **B:** Aliquots of reactions shown in A were resolved in a 4% nondenaturing gel. Positions of splicing complex B and U2/U4/U5/U6 snRNP:5′SS RNA complex are indicated. **C:** Top section of the gel shown in B was UV irradiated, the segment containing 5′SS:U2/U4/U5/U6 snRNP complex and splicing complex B were excised from the gel, and resolved in a second 10% SDS gel. Positions of the p220:5′SS RNA crosslink (arrow) and of the 200-kDa size marker are indicated.

at 30 °C (Fig. 5B, lanes 3–6) and its identity is currently under investigation. In the presence of Ad13 RNA, U2 snRNP and a number of protein factors bind to the 3′SS RNA to form splicing complex A (Konforti & Konarska, 1995). Upon addition of the 5′SS RNA, this complex is converted to splicing complex B by joining of U4/U5/U6 snRNP (Fig. 5B, lane 3, 4). The identity of this complex is based, in part, on its snRNP composition and its co-migration with authentic complex B formed in the *cis*-splicing reaction (data not shown). Furthermore, conditions allowing for the formation of this complex coincide with those required for *trans*-splicing (Fig. 5A,B, lanes 3, 4). To confirm the identity of this complex B as that of the functional intermediate in the spliceosome assembly pathway, we performed a chase experiment. *Trans*-splicing reaction was incubated for 30 min at 30 °C to allow for complex B to form (Fig. 5B, lane 3), at which time a 1000-fold excess of unlabeled 5′SS RNA oligo was added, and the incubation continued for additional 90 min (Fig. 5B, lane 7). Under these conditions, labeled 5′SS RNA present in complex B does not exchange with the unlabeled oligo added during the chase, because the level of splicing intermediates and products formed is similar to that found in the standard reaction (Fig. 5A, lanes 4, 7). Under the same conditions, the amount of labeled complex B decreases as it proceeds into splicing (Fig. 5B, lanes 4, 7). In reactions in which a 500-fold excess of unlabeled 5′SS RNA oligo was included from the beginning of incubation, no complex B or splicing products could be detected after 30 or 120 min (Fig. 5A,B, lanes 8, 9).

Having identified the complex formed in *trans*-splicing reactions as the authentic functional splicing complex B, we tested whether the 5′SS RNA in this complex interacts with p220 as in U2/U4/U5/U6 snRNP:5′SS RNA complex. The top portion of the nondenaturing gel shown in Figure 5B was irradiated with 254 nm UV light and the gel section containing U2/U4/U5/U6 snRNP:5′SS RNA and spliceosome B complexes was resolved in a 10% SDS gel. A prominent p220 crosslink is present in both U2/U4/U5/U6 snRNP:5′SS RNA and spliceosome B complexes (Fig. 5C, lanes 1–4). Thus, p220 interacts closely with the 5′SS RNA within the functional splicing complex B.

The effect of G+1 and U+2 modifications on pre-mRNA splicing

Thus far, we have shown that certain modifications of the GU dinucleotide at the 5′ end of the intron affect recognition of the 5′SS RNA and formation of spliceosome-like complexes. *Trans*-splicing reactions using 5′SS RNA oligos containing individual nucleotide modifications were performed to correlate the 5′SS RNA requirements for spliceosome assembly and splicing activity. In agreement with the results of the binding experiments (Fig. 4B; Table 1), introduction of the large methyl or iodo groups at position 5 of uridine within the GU dinucleotide (U+2 → rT and U+2 → 5IdU) reduced splicing to 48 and 50% of that of the wt 5′SS RNA oligo, respectively (Fig. 6, lanes 4, 5). In contrast, a smaller fluoro group placed at the analogous position (U+2→5FdU) had no major effect on splicing efficiency (85% of wt 5′SS RNA, Fig. 6, lane 6). Substitution of G residues with inosine (G−1→I, G+1→I, and G+5→I) resulted in a stimulation of splicing (138, 122, and 140% of wt, respectively, Fig. 6, lanes 8–10). However, consistent with previous studies (Aebi et al., 1987; Siliciano & Guthrie, 1988), splicing of the G+1 → C 5′SS RNA was blocked at the first step of the reaction, leading to

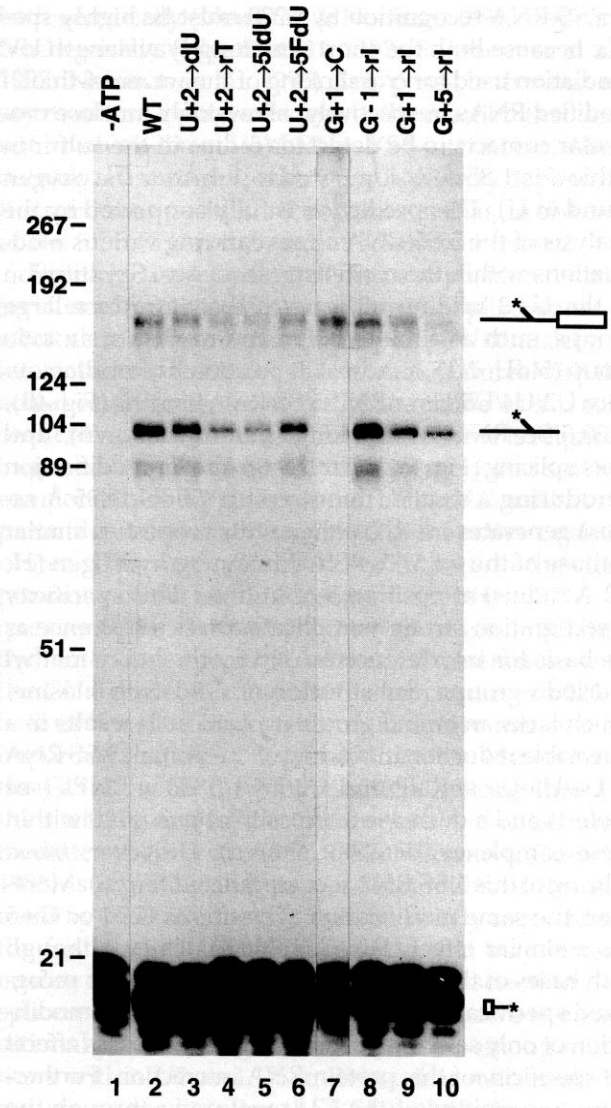

FIGURE 6. Effect of G+1 and U+2 modifications on *trans*-splicing. Splicing reactions were preincubated for 30 min at 30 °C in the presence of Ad13 RNA, followed by addition of the 3′ end-labeled 5′SS RNA and incubation continued for 2 h. RNA products were resolved in a 10% polyacrylamide/8 M urea gel. Splicing reactions containing wt 5′SS RNA were incubated in the absence (lane 1) or presence of ATP (lane 2). Modified 5′ SS RNA oligos as indicated were incubated under splicing conditions (lanes 3–10). The position of branched intron–exon intermediate, branched intron, and free unreacted 5′SS RNA oligo are indicated. Note that lanes 9 and 10 were underloaded.

the accumulation of the branched intron/3′ exon intermediate (Fig. 6, lane 7). Thus, certain modifications of the 5′SS (e.g., inosine substitutions at positions G−1, G+1, and G+5) reduce 5′SS:snRNP complex formation while not decreasing splicing efficiency. It appears that, under the conditions used, snRNP complex assembly is not limiting for splicing catalysis. However, other modifications (e.g., U+2→rT and U+2→5IdU), in addition to reducing snRNP complex formation, also affect splicing efficiency. Further studies will be necessary to determine whether spliceosome rearrangement or the first step of splicing is limiting in this case.

DISCUSSION

Sequence specificity of the 5′SS-dependent association of snRNPs

Although both RNA:RNA and protein:RNA interactions are known to be required for assembly of the spliceosome and progression of splicing, the contribution of individual elements of the spliceosome to this process is not understood. Although initial recognition of the 5′SS involves base pairing with the 5′ end of U1 snRNA (Zhuang & Weiner, 1986; Séraphin et al., 1988; Siliciano & Guthrie, 1988), this interaction is subsequently replaced by association with U5 and U6 snRNAs in the U4/U5/U6 snRNP complex. To study interactions between the 5′SS and the spliceosome components, we have employed a simple in vitro assay in which an RNA oligo comprising the 5′SS consensus sequence, the 5′SS RNA, induces specific assembly of spliceosome-like complexes and, in the presence of another RNA containing the 3′SS region, forms functional splicing complexes and undergoes *trans*-splicing. In this system, both the spliceosome assembly and *trans*-splicing assays are conducted in the presence of the 5′SS DNA oligo, which blocks the 5′ end of U1 snRNA and allows for the efficient association of the 5′SS RNA oligo with U4/U5/U6 and U2/U4/U5/U6 snRNP complexes (Konforti et al., 1993; Konforti & Konarska, 1995). The specificity of this interaction correlates well with the 5′SS consensus sequence derived from a compilation of all naturally occurring 5′ splice sites (Konforti & Konarska, 1994). In particular, mutations at the first two positions of the intron, G+1 and U+2, affect the interaction of the 5′SS RNA with U2/U4/U5/U6 snRNP complexes (~8% of the wt oligo binding) most dramatically. The GU dinucleotide at the 5′ end of introns represents the most highly conserved element within the 5′SS consensus sequence of nuclear mRNA precursors, being found in almost 100% of known 5′SS. For comparison, two other highly conserved Gs at positions −1 and +5 occur in 81 and 85% of mammalian 5′SS, whereas U at position +6 is found only in 45% of known cases (Senapathy et al., 1990).

Current understanding of the structure of the spliceosomal catalytic center explains some of the requirements for the 5′SS RNA sequence. Nucleotides surrounding position G+5 of the intron were shown to interact with U6 snRNA by base pairing with the highly conserved ACAGAG element of U6 snRNA (Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993). Consistent with these findings, the 5′SS RNA oligo present in U4/U5/U6 or U2/U4/U5/U6 snRNP complexes can be crosslinked to U6 snRNA due to base pairing involving the 3′ portion of the oligo (positions G+5, U+6, A+7, and T+8). In addition, the central loop in U5 snRNA was found to interact with exon sequences (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Sontheimer & Steitz, 1993; Newman et al., 1995),

providing a possible exon binding site within the spliceosome. Finally, a non-Watson–Crick interaction between the G residues flanking the intron has been postulated (Parker & Siliciano, 1993; Scadden & Smith, 1995). However, the most highly conserved element in the 5′SS sequence, the GU dinucleotide at the 5′ end of the intron, has not been implicated previously in any specific recognition event by the spliceosome components.

p220 interacts with the GU dinucleotide at the 5′ splice site

Here we present evidence that the GU element is recognized by p220, a protein component of the U5 snRNP, a human analogue of the yeast Prp8 protein (Anderson et al., 1989; Garcia-Blanco et al., 1990; Whittaker et al., 1990). The 5′SSRNA:p220 crosslink is detected in the context of U4/U5/U6 and U2/U4/U5/U6 snRNP complexes, suggesting that U4/U5/U6 snRNP in the absence of U2 snRNP is sufficient to promote p220:5′SS RNA interaction. In addition, the p220 crosslink is found in functional complex B, an intermediate complex in the spliceosome assembly pathway. The 5′SS RNA:p220 interaction seems to require intact snRNP particles because the p220 crosslink is not detected in reactions pre-treated with micrococcal nuclease (data not shown). p220 represents the only protein in the large multi-snRNP complex that crosslinks to the 5′SS RNA, confirming the high specificity of this interaction. The crosslink between the 5′SS RNA and p220 is located within the GU dinucleotide at the 5′ end of the intron. Although crosslinking of the unmodified guanosine at G+1 position was detected upon irradiation with 254 nm UV light, direct and specific contact at U+2 position was demonstrated using 4-thioU-substituted RNA oligo irradiated with 365 nm light. Crosslinks between Prp8/p220 and pre-mRNA reported previously for both yeast and mammalian systems (Garcia-Blanco et al., 1990; Whittaker & Beggs, 1991) have demonstrated interactions with the branch site, polypyrimidine tract, and the 5′SS region (Wyatt et al., 1992; MacMillan et al., 1994; Teigelkamp et al., 1995; Umen & Guthrie, 1995). The crosslinks observed within the 5′SS were located at position −2 in the exon using 4-thioU-modified pre-mRNA (Wyatt et al., 1992). Recently, an extensive mapping of Prp8 interaction with pre-mRNA found 4-thioU-specific crosslinks at positions −8, −2, and −1 in the 5′ exon sequences, but not at position +4 in the intron (Teigelkamp et al., 1995). However, crosslinking of 4-thioU at position +2 in the intron was not tested in that study. Because of the significant functional similarity between Prp8 and p220, it is expected that p220 also contacts 5′ exon upstream of the 5′ splice site. Due to the lack of conservation of exon sequences, these contacts cannot be sequence specific, suggesting that the main recognition signal for the Prp8/p220:5′SS interaction is localized within the GU dinucleotide.

5′SS RNA recognition by p220 must be highly specific because both the short- and long-wavelength UV irradiation used for crosslinking of the wt and 4-thioU-modified RNAs, respectively, allows only for close molecular contacts to be detected (radius of the sulfur in 4-thioU is 1.85 Å as compared to 1.4 Å for the oxygen found in U). This prediction is fully supported by the analysis of the 5′SS RNA oligos carrying various modifications within the consensus sequence. Substitution of the U+2 uridine with bases that introduce large groups, such as a methyl (T, 2.0 Å radius) or an iodo group (5IdU, 2.15 Å radius) at position 5 of uridine, reduce U2/U4/U5/U6 snRNP:5′SS RNA binding (Fig. 4B), p220:5′SS RNA crosslinking (data not shown), and *trans*-splicing (Fig. 6). In contrast, a similar modification introducing a smaller fluoro group (5FdU, 1.35 Å radius) generates an RNA oligo with properties similar to those of the wt 5′SS RNA containing hydrogen (H, 1.2 Å radius) at position 5 of uridine. This specificity of recognition strongly implicates steric hindrance as the basis for interference caused by the bulky methyl and iodo groups. Substitution of G+1 with inosine, which lacks an amino group at position 2, results in a detectable reduction in binding of the mutant 5′SS RNA to U4/U5/U6 snRNP and U2/U4/U5/U6 snRNPs (see Table 1) and a decrease in crosslinking to p220 within these complexes (data not shown). However, *trans*-splicing of this 5′SS RNA was stimulated (Fig. 6). Moreover, the same modification at positions G−1 or G+5 has a similar effect (Fig. 6; Table 1). Thus, although both bases of the GU dinucleotide appear to be recognized specifically by p220 in the spliceosome, modification of only selected positions within the bases affects the specificity of this protein:RNA interaction. Furthermore, recognition of the GU must occur through the bases because substitution of the ribose moiety with deoxyribose or 2′-O-methyl ribose at the same positions in the sequence does not significantly affect binding of the 5′SS RNA oligo to snRNP complexes (data not shown).

Implications for the catalytic center of the spliceosome

U6 snRNA has been previously shown to interact with the 5′SS sequence during splicing. Using UV crosslinking methods, we have detected an analogous interaction within the spliceosome-like complexes formed in the presence of the 5′SS RNA (Konforti et al., 1993). Similarly, the 5′SS RNA carrying 4-thioU modification at U+6 position crosslinks to U6 snRNA. In contrast, the U+2 → 4-thioU 5′SS RNA crosslinks to p220, but not to U6 snRNA, suggesting that crosslinking of different 5′SS RNA oligos reflects distinct interactions involving the 5′SS. Interestingly, we have never detected a crosslink between the 5′SS RNA and U5 snRNA. Such an interaction during splicing was demonstrated

both genetically (Newman & Norman, 1991, 1992) and biochemically (Wyatt et al., 1992; Sontheimer et al., 1993; Newman et al., 1995). It is possible that our assay monitors a different stage in the reaction that does not involve a direct U5 snRNA:5′SS interaction. Alternatively, base pairing with U5 snRNA may depend on the particular positioning of the exon that, in the case of the 5′SS RNA oligo, is not stably maintained.

Together, these findings allow us to position the major sites of 5′SS recognition within the spliceosome. It is not clear at the moment if the p220:GU dinucleotide interaction represents or shares some features with the actual catalytic center of the spliceosome or if a subsequent rearrangement of the spliceosome is required to generate a functional catalytic center. The snRNA components of the spliceosome, U2 and U6 snRNAs in particular, are thought to comprise the active catalytic center. This suggestion is based in part on the close proximity of the highly conserved, specific snRNA sequences to the site of actual catalysis of splicing. Thus, the remarkable specificity of the p220 interaction with the GU dinucleotide and the close proximity of p220 to both splice sites during the reaction also makes it an important element of the catalytic center. Mutations of the GU allow for the progression of splicing through the first step and lead to accumulation of lariat intermediates (Aebi et al., 1986, 1987; Siliciano & Guthrie, 1988), suggesting that proper recognition of this element by p220 may not by itself be strictly required for the first transesterification reaction, but may instead ensure the proper fidelity of substrate selection and spliceosome assembly where p220 may serve as an additional proofreading factor. However, among mutations within U2 and U6 snRNAs that map near the sequences used to align the 5′SS with the branch site, some block splicing before the first step, whereas others permit the first but not the second step of splicing to proceed (Fabrizio & Abelson, 1990; McPheeters & Abelson, 1992). Similarly, although most of the known PRP8 alleles prevent splicing catalysis, at least one, prp8-101, blocks splicing after the first step (Brown & Beggs, 1992; Umen & Guthrie, 1995). Thus, both mutations that affect the p220:5′SS interaction and those that modify U2 and U6 snRNAs near the catalytic center of the spliceosome can cause a similar defect, namely, uncoupling of the two steps of splicing. Therefore, the possibility that p220 is involved in the catalysis of splicing should be considered. Further elucidation of p220 function will be essential to our understanding of the mechanism of splicing.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems 390 synthesizer. Riboinosine phosphoramidite was obtained from ChemGenes (Waltham, Massachusetts). Ribothymidine phosphoramidite (generous gift of S. Strobel and T. Cech) was synthesized as described (Strobel et al., 1994). Sulfur-unprotected 4-thiouridine phosphoramidite was prepared by standard 5′-O-dimethoxytritylation, followed by selective 2′OH protection with *t*-butyldimethylsilyl chloride (Usman et al., 1987). The nucleoside was then converted to the phosphoramidite using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (Scaringe et al., 1990). All other phosphoramidites were from Glen Research (Sterling, Virginia).

5′ and 3′ end-labeling of oligos was performed as described before (Konforti & Konarska, 1994). 4-thioU-containing oligos were affinity-purified in 20% polyacrylamide/8 M urea gels containing 0.1 mg/mL of *N*-acryloylaminophenyl mercuric chloride (Igloi, 1988).

A_6G/p*GUAAGUAdTdC_3 oligo used in experiments shown in Figures 3B and 5 was prepared by 5′ end-labeling of the /GUAAGUAdTdC_3 oligo and subsequent ligation to A_6G using T4 RNA ligase (Boehringer Mannheim). For the experiments in Figure 5, the 5′SS RNA oligo was further 5′ end-phosphorylated to reduce its participation in competing RNA ligase reactions in the nuclear extract (Konforti & Konarska, 1995). Oligos for experiments in Figure 6 were prepared by ligation of the 3′ end-labeled 5′SS RNA (AAG/GUAAGUA dTdC^*_3) to A_6 using T4 RNA ligase.

In vitro binding and trans-splicing assays

Binding reactions (10 μL) were performed as described previously (Konforti & Konarska, 1994). snRNP-free supernatants were obtained by centrifugation of nuclear extracts for 1.5 h at 400,000 × *g*. *Trans*-splicing reactions (15μL) were performed as described (Konforti & Konarska, 1995). Electrophoresis was conducted under conditions where the unbound/unreacted 5′SS RNA oligo remained in the gel to allow for quantitation of efficiency of complex formation/*trans*-splicing reaction. All gels were quantified using a Molecular Dynamics phosphorimager.

Crosslinking experiments

Binding reactions (10 μL) were transferred to a microtiter plate embedded in ice and irradiated three times for 5 min with a 254 nm UV lamp (Spectroline) placed directly on the uncovered plate. Samples containing 4-thioU RNA oligos were irradiated using a 365 nm UV lamp (Spectroline) placed on the covered plate. When indicated, UV crosslinking was followed by addition of RNase T1 (2 units), or RNase A (2 μg) and incubation continued for 20 min at 30 °C. Samples were resolved in a 15% polyacrylamide SDS gel and products visualized by autoradiography.

For the experiments shown in Figure 1C and D, snRNP complexes formed in binding reactions were UV irradiated for 15 min and then resolved in a 4% nondenaturing gel. Complexes shown in Figure 5 were resolved in a 4% nondenaturing gel and then UV irradiated for 6 min (2 × 3 min). Gel sections containing snRNP complexes were cut out and resolved in a 10% polyacrylamide SDS gel.

An antibody raised against a 50-kDa C-terminal fragment of human p220, the generous gift of M.J. Moore and P.A. Sharp, was used for immunoprecipitation experiments. Anti-

p220 or pre-immune sera were coupled to Protein A-trisacryl beads (Pierce) in NET-2 buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.05% Nonidet P40) for 2 h on ice and washed twice with NET-2 buffer. The completeness of RNase A treatment of UV irradiated binding reactions was monitored by northern hybridization using snRNA-specific probes. Reactions were incubated with the antibody-coupled beads for 3 h on ice in the presence of 1 mg/mL of heparin, and rinsed twice with NET-2 buffer. Bound material was resolved in a 15% polyacrylamide SDS gel.

Wet SDS gel slices containing p220 crosslinks were digested with proteinase K (0.3 mg) for 1 h at 37 °C in 30 μL of 10 mM Tris, pH 7.5, 10 mM NaCl, 0.1% SDS. Subsequent RNase A (2 μg) and RNase T1 (2 U) digestions were performed for 30 min at 30 °C in 50 mM Tris, pH 7.7. Nuclease P1 digestions were for 1 h at 50 °C in 20 mM Na acetate, pH 5.2. Digestion products were resolved in a 20% polyacrylamide/8 M urea gel.

ACKNOWLEDGMENTS

We are grateful to M. Moore, Brandeis University, and P.A. Sharp, MIT, for the gift of the p220 antibody; to S. Strobel and T. Cech, University of Colorado, Boulder, for the riboT phosphoramidite; and to K. Watanabe, Sloan Kettering Institute, for the use of his facilities for the synthesis of the 4-thioU phosphoramidite. We thank L. Dailey and K. Hall for helpful discussions and critical reading of the manuscript. J.L.R. is a John Calvert Eisenstein Fellow and was supported partially by a fellowship from Universidad Nacional Autónoma de México. B.B.K. was supported by a Helen Hay Whitney Foundation postdoctoral fellowship. This work was supported by the Markey Charitable Trust Scholar, a Monique Weill-Caulier Award, and an NIH grant GM49044 to M.M.K.

Received January 19, 1996; returned for revision February 8, 1996; revised manuscript received February 14, 1996

REFERENCES

Aebi M, Hornig H, Padgett RA, Reiser J, Weissmann C. 1986. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. *Cell 47*:555-565.

Aebi M, Hornig H, Weissmann C. 1987. 5′ cleavage site in eukaryotic pre-mRNA splicing is determined by the overall 5′ splice region, not by the conserved 5′ GU. *Cell 50*:237-246.

Anderson GJ, Bach M, Lührmann R, Beggs JD. 1989. Conservation between yeast and man of a protein associated with U5 snRNP. *Nature 342*:819-821.

Bergstrom DE, Leonard NJ. 1972. Photoreaction of 4-thio-uracil with cytosine. Relation to photoreactions in *E. coli* tRNA. *Biochemistry 11*:1-9.

Brown JD, Beggs JD. 1992. Roles of PRP8 protein in the assembly of splicing complexes. *EMBO J 11*:3721-3729.

Fabrizio P, Abelson J. 1990. Two domains of yeast U6 snRNA required for both steps of nuclear precursor messenger RNA splicing. *Science 250*:404-409.

Garcia-Blanco MA, Anderson GJ, Beggs J, Sharp PA. 1990. A mammalian protein of 220 kD binds pre-mRNAs in the spliceosome: A potential homologue of the yeast PRP8 protein. *Proc Natl Acad Sci USA 87*:3082-3086.

Ge H, Manley JL. 1990. A protein factor, ASF, controls cell-specific alternative splicing of SV40 early pre-mRNA in vitro. *Cell 62*: 25-34.

Hall KB, Konarska MM. 1992. The 5′ splice site consensus RNA oligonucleotide induces assembly of U2/U4/U5/U6 small nuclear ribonucleoprotein complexes. *Proc Natl Acad Sci USA 89*:10969-10973.

Igloi GL. 1988. Interaction of tRNAs and of phosphorothioate-substituted nucleic acids with an organomercurial. Probing the chemical environment of thiolated residues by affinity electrophoresis. *Biochemistry 27*:3842-3849.

Kandels-Lewis S, Séraphin B. 1993. Role of U6 snRNA in 5′ splice site selection. *Science 262*:2035-2039.

Kohtz JD, Jamison SF, Will CL, Zuo P, Lührmann R, Garcia-Blanco MA, Manley JL. 1994. Protein–protein interactions and 5′ splice site recognition in mammalian mRNA precursors. *Nature 368*: 119-124.

Konforti BB, Konarska MM. 1994. U4/U5/U6 snRNP recognizes the 5′ splice site in the absence of U2 snRNP. *Genes & Dev 8*:1962-1973.

Konforti BB, Konarska MM. 1995. A short 5′ splice site RNA oligo can participate in both steps of splicing in mammalian extracts. *RNA 1*:815-827.

Konforti BB, Koziolkiewicz MJ, Konarska MM. 1993. Disruption of base pairing between the 5′ splice site and the 5′ end of U1 snRNA is required for spliceosome assembly. *Cell 75*:863-873.

Krainer AR, Conway GC, Kozak D. 1990. The essential pre-mRNA splicing factor SF2 influences 5′ splice site selection by activating proximal sites. *Cell 62*:35-42.

Lemaigre Dubreuil Y, Expert-Bezançon A, Favre A. 1991. Conformation and structural fluctuations of a 218 nt long rRNA fragment: 4-thiouridine as an intrinsic photolabelling probe. *Nucleic Acids Res 19*:3653-3660.

Lesser CF, Guthrie C. 1993. Mutations in U6 snRNA that alter splice site specificity: Implications for the active site. *Science 262*:1982-1988.

MacMillan AM, Query CC, Allerson CR, Chen S, Verdine GL, Sharp PA. 1994. Dynamic association of proteins with the pre-mRNA branch region. *Genes & Dev 8*:3008-3020.

McPheeters DS, Abelson J. 1992. Mutational analysis of the yeast U2 snRNA suggests a structural similarity to the catalytic core of group I introns. *Cell 71*:819-831.

Moore MJ, Query CC, Sharp PA. 1993. Splicing of precursors to mRNA by the spliceosome. In: Gesteland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 303-357.

Newman A, Norman C. 1991. Mutations in yeast U5 snRNA alter specificity of 5′ splice site cleavage. *Cell 65*:115-123.

Newman AJ, Norman C. 1992. U5 snRNA interacts with exon sequences at 5′ and 3′ splice sites. *Cell 68*:743-754.

Newman AJ, Teigelkamp S, Beggs JD. 1995. snRNA interactions at 5′ and 3′ splice sites monitored by photoactivated crosslinking in yeast spliceosomes. *RNA 1*:968-980.

Parker R, Siliciano PG. 1993. Evidence for an essential non-Watson-Crick interaction between the first and last nucleotides of a nuclear pre-mRNA intron. *Nature 361*:660-662.

Pinto AL, Steitz JA. 1989. The mammalian analogue of the yeast PRP8 splicing protein is present in the U4/5/6 small nuclear ribonucleoprotein particle and the spliceosome. *Proc Natl Acad Sci USA 86*: 8742-8746.

Sawa H, Abelson J. 1992. Evidence for a base-pairing interaction between U6 snRNA and the 5′ splice site during the splicing reaction in yeast. *Proc Natl Acad Sci USA 89*:11269-11273.

Scadden ADJ, Smith CWJ. 1995. Interactions between the terminal bases of mammalian introns are retained in inosine-containing pre-mRNAs. *EMBO J 14*:3236-3246.

Scaringe SA, Francklyn C, Usman N. 1990. Chemical synthesis of biologically active oligoribonucleotides using beta-cyanoethyl protected ribonucleoside phosphoramidites. *Nucleic Acids Res 18*: 5433-5441.

Senapathy P, Shapiro MB, Harris NL. 1990. Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project. *Methods Enzymol 183*:252-278.

Séraphin B, Kretzner L, Rosbash M. 1988. A U1 snRNA:pre-mRNA base pairing interaction is required early in yeast spliceosome assembly but does not uniquely define the 5′ cleavage site. *EMBO J 7*:2533-2538.

Siliciano PG, Guthrie C. 1988. 5′ splice site selection in yeast: Genetic alterations in base-pairing with U1 reveal additional requirements. *Genes & Dev 2*:1258-1267.

Sontheimer EJ, Steitz JA. 1993. The U5 and U6 snRNAs as active site components of the spliceosome. *Science 262*:1989–1996.

Stolow DT, Berget SM. 1991. Identification of nuclear proteins that specifically bind to RNAs containing 5′ splice sites. *Proc Natl Acad Sci USA 88*:320–324.

Strobel S, Cech TR, Usman N, Beigelman L. 1994. The 2,6-diaminopurine riboside·5-methylisocytidine wobble base pair: An isoenergetic substitution for the study of G·U pairs in RNA. *Biochemistry 33*:13824–13835.

Teigelkamp S, Newman AJ, Beggs JD. 1995. Extensive interactions of PRP8 protein with the 5′ and 3′ splice sites during splicing suggest a role in stabilization of exon alignment by U5 snRNA. *EMBO J 14*:2602–2612.

Umen JG, Guthrie C. 1995. A novel role for a U5 snRNP protein in 3′ splice site selection. *Genes & Dev 9*:855–868.

Usman N, Ogilvie KK, Cedergreen RJ. 1987. Automated chemical synthesis of long oligonucleotides using 2′-O-silylated ribonucleoside 3′-O-phosphoramidites on a controlled-pore glass support: Synthesis of a 43-nt sequence similar to the 3′-half molecule of a *E. coli* formylmethionine tRNA. *J Am Chem Soc 109*:7845–7854.

Whittaker E, Beggs JD. 1991. The yeast PRP8 protein interacts directly with pre-mRNA. *Nucleic Acids Res 19*:5483–5489.

Whittaker E, Lossky M, Beggs JD. 1990. Affinity purification of spliceosomes reveals that the precursor RNA processing protein PRP8, a protein in the U5 snRNP, is a component of yeast spliceosomes. *Proc Natl Acad Sci USA 87*:2216–2219.

Wyatt JR, Sontheimer EJ, Steitz JA. 1992. Site-specific cross-linking of mammalian U5 snRNP to the 5′ splice site before the first step of pre-mRNA splicing. *Genes & Dev 6*:2542–2553.

Zahler AM, Neugebauer KM, Lane WS, Roth MB. 1993. Distinct functions of SR proteins in alternative pre-mRNA splicing. *Science 260*:219–222.

Zhuang Y, Weiner AM. 1986. A compensatory base change in U1 snRNA suppresses a 5′ splice site mutation. *Cell 46*:827–835.